THE RÔLE OF NITROGEN IN PLANT-METABOLISM.

Part iii.—The Distribution of Nitrogen in Acacia Seeds.

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As a large stock of the seeds of *Acacia pycnantha* was made available to the writer some time ago, the opportunity was taken to make an experimental study of the proteins and other nitrogen compounds contained in them. The seeds were in a mature condition and had been kept about two years in stock.

Preliminary Examination.—For the purpose of analysis, a quantity of the seeds, with their tightly adhering black tests left on, was crushed as finely as possible in a small mill. A portion was dried at 100° C., to ascertain the amount of moisture; the residue was incinerated and the weight of the ash noted. In another portion, the nitrogen was estimated by Kjeldahl's method. As it would be a matter of very great difficulty to separate the different parts of these seeds mechanically, it was expected that a number of different proteins would be dissolved together in the extracts. The greater part would represent the reserve proteins of the seed; and a smaller amount would be extracted from the protoplasm of the embryonic tissues.

A quantity of the finely powdered seeds was first extracted with distilled water, until very little more was dissolved. The extract was filtered clear, and the insoluble residue then further extracted as completely as possible with a 10% solution of sodium chloride. The residue obtained after filtration was next treated with alcohol of 80% strength, the alcoholic extract was filtered, and the residue then examined.

A considerable amount of nitrogen still remained in the insoluble residue of seedmeal after all these solvents had been used. The nature of this nitrogen is still unknown, although it most likely occurs in the form of protein also. The fact that, when the residue is dried and ground still finer, more protein is extracted, shows that some cells had resisted disintegration and still enclosed part of the protein. There is no doubt that tannins take an important part by combining with the protein, and forming an insoluble compound, for tannins are found in considerable quantities in the skins of Acacia seeds. Part of the nitrogen in the residue can be removed as protein by dilute alkalies and acids; but it is known, however, that these solvents alter the chemical nature of the proteins by combining with them, and that the original protein cannot again be restored. These reagents were, therefore, not applied in this case.

The distilled water and salt-extracts were each measured. One portion of each was used for the estimation of the total amount of nitrogen, and in another portion the proteins were precipitated by tannic acid; the tannic precipitates were dropped into a flask, and the nitrogen determined by Kjeldahl's method as protein-nitrogen. The results of these determinations are stated in the following table:—

TABLE I.—ACACIA SEEDS.

Water driven off at 100°C.	11·58 %
Ash	3·34
Organic constituents (by difference)	85·08
	100·000

NITROGEN IN THE VARIOUS EXTRACTS.

	in 100 gms. seeds	% of total nitrogen
Total nitrogen in the seeds	4·51 %	100
Protein N soluble in water	1·20	26·6
insol. in water, sol. in 10 % salt	0·60	13·3
sol. in alcohol 80 %	0·00	0·0
insol. in water and salt—unextracted	0·68	15·1
N of other forms sol. in water, not pptd. by [illegible]	2·03	45·0

It is noteworthy that a comparatively large amount of the protein is dissolved out by water alone. According to Osborne, it may contain proteoses, albumins, and globulins. From the amount of ash present, it is apparent that the addition of distilled water to the seeds is equivalent to a dilute saline solution, so that more than the real water-soluble proteins is obtained in the extract.

The alcoholic extract was treated with ether, but no precipitation followed, showing the entire absence of alcohol-soluble proteins.

It was observed that the clear saline extracts, after standing for some time, increased in acidity, and gradually deposited part of the proteins in an insoluble form. This appears to be caused by the combination of the acid and protein, forming an insoluble salt.

Osborne* has shown that a large number of proteins in plants possess more pronounced basic properties than acidic. They can easily form salts with the organic acids of the extracts. The salt-extracts were all found to be distinctly acid to litmus, and much more acid in reaction towards phenolphthalein. Now these insoluble salts of protein, formed during the extraction, may be redissolved by careful titration with sodium hydroxide, to the phenolphthalein neutral point. We then obtain the sodium salt of the organic acid, and the protein is set free in a form soluble in the saline solution. This, by the careful titration of the salt-extracts

(*) Journ. Amer. Chem. Soc. 1902, p. 39.

containing protein in suspension, much of the latter may be redissolved, and still any increase in the concentration of hydroxyl ions is avoided. This in no way resembles the conditions of extraction by dilute alkalies.

The following comparative tests were made on the solubility of the seed-proteins in various concentrations of sodium chloride, sodium hydroxide, and the two together.

Method.—Two grammes of finely-powdered seeds were extracted, for 24 hours, with the solvents stated in Table ii., and frequently shaken. All the extracts reacted acid to phenolphthalein, but alkaline to litmus. An equal volume of each clear filtered extract was then transferred to a centrifuge tube, and precipitated by the addition of 6 drops of salicylsulphonic acid. After spinning, the supernatant fluids were decanted, and the deposits were washed free from acid (neutral to phenolphthalein).

To each tube was then added 20 cc. of 1% saline solution, and the opalescent fluids titrated with sodium hydroxide. After titration, each was transferred to a Kjeldahl flask, and this nitrogen determined. The results are given in the table below:—

TABLE ii.

No.	Solvent		Titration $\frac{N}{100}$ NaOH	N $\frac{N}{10}$ acid
	NaOH	NaCl		
1	0·1 %		11 cc.	7·8 cc.
2	0·2		28	11·6
3	0·1	1 %	11	8·0
4	0·2	1	18	9·1
5	0·1	2	11	9·0
6	0·2	2	14	9·5
7	0·1	5	15	10·8
8	0·2	5	14	10·0
9	0·1	7	14	10·4
10	0·2	7	15	9·5
11	0·1	10	14	9·9
12	0·2	10	14	lost
13		1	4	5·2
14		5	4	7·0
15		10	4	7·9

Nos. 13, 14 and 15 show the relative amounts dissolved by salt alone, of which 10% is the best solvent. Almost the same amount of protein is dissolved by 10% salt as by 0·1%alkali. The partially neutralised extracts in the whole series, from 1% to 10%, contain more than the salt extracts alone. The maximum protein is obtained in No. 2, by using 0·2% alkali alone.

Preparation of Solutions for Analysis.—Since little is to be gained by first extracting, with water, those proteins which are also soluble in salt-solutions, the extracts were always made by adding sodium chloride solution directly to the powdered seeds.

By extracting 20 gms. of seeds with 1 litre of 10% sodium chloride solution for 1 day, an extract was obtained in which the protein-content was determined by precipitation with tannic acid, and the estimation of nitrogen in the precipitate. The residue of seeds was again extracted with a second litre of salt-solution, and the amount of protein-nitrogen estimated as before. By successive treatments in this way, until no protein was contained in the final solution, the maximum quantity of protein capable of being extracted by sodium chloride solution was ascertained. The results were as follows:—

1st	Extraction	yielded	76	%	of the whole	extractable protein.
2nd	,,	,,	14	,,	,,	,,
3rd	,,	,,	6	,,	,,	,,
4th	,,	,,	2	,,	,,	,,
5th	,,	,,	1	,,	,,	,,
6th	,,	,,	0·6	,,	,,	,,
7th	,,	,,	0·3	,,	,,	,,

In consequence of the above, the numerous single extracts made for various experiments, give results which are not comparable with one another.

In order to study the behaviour of these proteins towards the different reagents, an extract was made from 100 gms. of

seeds in 10% sodium chloride solution, which contained 3·05 gms. of nitrogen.

(*a*) To 50 cc. of the extract, were added 10 drops of a 2% solution of acetic acid, then heated to boiling in the water-bath for some time. The coagulated protein was filtered; the filtrate was boiled for some time longer, and the small amount of precipitate obtained was added to the first. The coagulum, after washing with hot water, was Kjeldahled for nitrogen. The filtrate from the coagulated protein showed no biuret reaction, indicating absence of peptones. On titrating with alkali till neutral to phenolphthalein, no precipitate was observed.

(*b*) Fifty cc. of the extract were precipitated by a 5% tannic acid solution, and kept cool by standing in water; after spinning in a centrifuge, and washing with diluted reagent, the nitrogen was estimated.

(*c*) Fifty cc. of the extract were diluted with water to 250 cc., and a rapid current of carbon dioxide passed through it for some time. The precipitate was separated in the centrifuge. The fluid, after further diluting with an equal volume of water, was again treated with the gas, when a small precipitate was obtained, which was added to the first, and the nitrogen estimated. The filtrate was further tested by boiling, when a coagulum formed, which was removed, and its nitrogen also estimated.

The results are stated below:—

TABLE iii.

	N gms.	% of the total N of seeds
Seeds, 100 gms. contain	4·51	100·
Salt-extract contains	3·05	67·6
(*a*) Coagulum by boiling	1·064	23·6
(*b*) Tannic acid ppt.	1·350	30·0
(*c*) CO_2 precipitate	0·851	18·9
Filtrate coagulated by heat	0·132	2·9
Filtrate not coagulated	2·041	45·3

A second extract was obtained as before, with 10% sodium chloride solution, and further precipitations made for comparison:

(*a*) Coagulation on boiling, (*b*) tannic acid ppt., as before,

(*c*) Trichloracetic acid added to the extract, which was then heated to boiling point, and filtered hot. The filtrate was then allowed to cool, whereupon it became cloudy, and deposited a further precipitate. On boiling once more, this deposit redissolved, and was again obtained on cooling. The nitrogen in each was estimated separately.

(*d*) Saturation with sodium chloride till the protein was salted out; after remaining some time with excess of salt still visible, it was separated by the centrifuge, and its nitrogen estimated. (*e*) Phosphotungstic acid was added to the filtrate from the tannic acid precipitation, after acidifying with sulphuric acid. In the protein-free filtrate, phosphotungstic acid precipitates the basic nitrogen compounds,—cholin, histidin, arginin, &c., if present.

The results are given in the following table:—

TABLE IV.

	N gms.	% of total N of seeds.
Seeds, 100 gms. contain.....	4·510	100·
Salt-extract contains..	1·670	37·0
(*a*) Protein coagulated by heat.....	0·448	9·9
(*b*) ,, pptd. by tannic acid.....	0·629	14·0
(*c*) ,, ,, by trichloracetic hot.....	0·516	11·45
Deposited on cooling after (*c*).....	0·075	1·65
(*d*) Protein salted out with saturated NaCl.	0·093	2·06
(*e*) Pptd. by phosphotungstic acid from tannin filtrate...	0·317	7·1

Taking tannic acid, which yields the maximum protein-precipitate as a standard, it is seen that the protein coagulated by boiling is in both experiments considerably less, in fact only about 75% of the tannic acid precipitate. This shows how imperfectly plant-proteins are coagulated, even on boil-

ing for a considerable time. The acid added no doubt plays a considerable part in the denaturing, for if an extract be previously neutralised to phenolphthalein with alkali, and then boiled, scarcely any coagulation takes place. When a 1% sodium chloride extract is heated very slowly, coagulation begins about 65° C., and the solution is distinctly acid. The protein has formed a compound with the acids during the extraction, and the conditions are then assured for heat-coagulation. The precipitate obtained by carbon dioxide in a dilute saline solution is now considered to be composed entirely of globulins. The extent of dilution necessary is of great importance. In this case, 10 vols. of water were required for complete separation. The nitrogen is equivalent to 63% of the tannic acid nitrogen.

Trichloracetic acid is usually stated as a precipitant for globulins, albumins, and proteoses. The latter, however, may be kept dissolved by boiling and filtering the liquid hot. The filtrate, after cooling, deposits these proteoses, which may then be separated from the perfectly cold liquid by filtration. This small deposit easily redissolved on boiling with water, and was as easily recovered by cooling. It amounted to 1·6% of the total nitrogen. By saturation of the extract with sodium chloride, a very small amount of protein is salted out. Finally, phosphotungstic acid was used to precipitate the basic constituents of the non-protein part. It was not used as a protein-precipitant.

A number of attempts were made to dialyse a solution of proteins obtained by salting out with ammonium sulphate, but it was not found an easy task to completely prevent the changes due to fungoid and bacterial contamination from taking place, and they had to be abandoned. The proteins on being salted out by complete saturation with ammonium sulphate, only partially went into solution again, when the precipitate was diluted with water. The figures obtained for albumins and globulins were, therefore, not reliable, and are omitted.

The following table gives an approximate view of the precipitation-limits with ammonium sulphate, of the proteins present in a 10% salt-extract.

TABLE V.—FRACTIONAL SATURATION WITH AMMONIUM SULPHATE.

No.	Salt extract.	Saturated Am. Sulp.	Water.	Result.
1	2 cc.	0 cc.	8 cc.	No ppt., faint opalescence
2	2	1	7	,, ,,
3	2	2	6	,, ,,
4	2	3	5	,, ,,
5	2	4	4	,, ,,
6	2	5	3	,, ,,
7	2	6	2	Pptn. begins, clear fluid
8	2	7	1	Large ppt. ,,
9	2	8	0	Larger ppt. ,,

Each of the nine tubes contained 10 cc. of fluid. Successively increasing amounts of ammonium sulphate were used, and the point observed at which incipient precipitation took place. This occurred in No. 7, with six-tenths saturation. With a lower concentration, no salting out occurred, and above this point the precipitation rapidly increased.

Quantitative Precipitation by Alcohol.—Alcohol precipitates globulins, albumins, proteoses, and peptones, in the order named. Animal-peptones are stated by Mann to be partly soluble, even in 96% alcohol, though Kühne and Chittenden observe that the precipitation is much more complete in the presence of salts. As plant-proteins vary considerably in the strength of alcohol required for their precipitation, the following series of experiments was designed to ascertain if any difference could be detected in the various fractions obtained by gradually increasing strengths of alcohol. For this purpose, a protein-extract was prepared with 5% sodium chloride, and filtered clear.

(*a*) Of this solution, 20 cc. were placed in each of eight centrifuge-tubes, and the calculated proportions of alcohol

and water added so that, maintaining the same volume of liquid throughout, the strength of spirit ranged from 10 to 80%. These tubes were allowed to stand over night to deposit, then spun in the centrifuge. After decantation of the superfluid, the deposits were transferred to small, accurately weighed centrifuge-tubes, washed once with alcohol of the same strength, followed by two washings with absolute alcohol, and finally with dry ether. The tubes were next dried carefully at 50° C. in the oven, finally at 110° to constant weight, and weighed under exact conditions. The contents of each tube were then shaken out into a weighed platinum crucible, and the tubes reweighed. The dry white powder was incinerated, and the weight of the ash determined. The weight of ash-free protein was thus obtained.

(*b* In a precisely similar series of duplicates precipitated under the same conditions, as nearly as possible, the moist deposits, after washing with alcohol of the same strength, were each transferred to a Kjeldahl flask, and the nitrogen estimated.

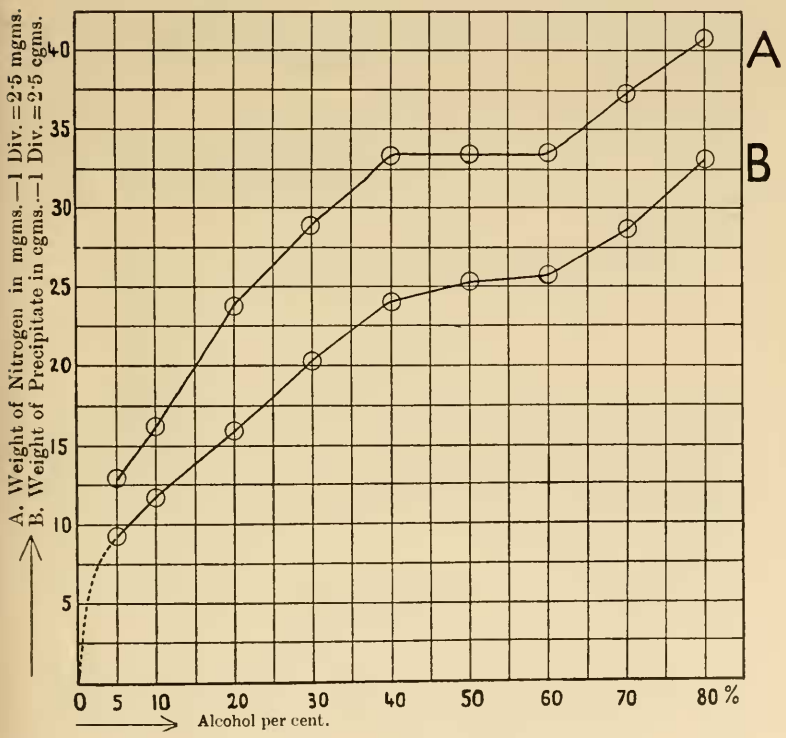
The details of these experiments are tabulated below:—

TABLE vi.

No.	Alcohol present.	Preparation.			Wt. of deposit.	Ash.	Ash. free protein.	Nitrogen.
		Extract	95% Alcohol	Water.				
	%	cc.	cc.	cc.	gm.	gm.	gm.	gm.
1	5	20	6·5	100	0·0916	...	0·0916	0·0131
2	10	20	13·5	93·5	0·1188	0·0020	0·1168	0·0164
3	20	20	27·0	80·0	0·1600	0·0024	0·1576	0·0241
4	30	20	40·5	66·5	0·2055	0·0024	0·2031	8·0289
5	40	20	54·0	53·0	0·2450	0·0050	0·2400	0·0335
6	50	20	67·5	39·5	0·2600	0·0072	0·2528	0·0347
7	60	20	81·0	26·0	0·2657	0·0088	0·2569	0·0335
8	70	20	94·5	13·5	0·3035	0·0166	0·2869	0·0375
9	80	20	107·0	0	0·3606	0·0296	0·3310	0·0407

These results are plotted in the accompanying curves, of which the ordinates represent—in A, the weights of nitrogen in milligrams; and in B, the weights of precipitate in centigrams. Abscissæ represent the percentages of alcohol present.

The nitrogen-curve, A, indicates the existence of more than one distinct protein. The first and least soluble protein has begun to precipitate with 5% alcohol, and, at this low concentration, nearly one-third of the total protein in the



extract is precipitated. From the initiation, up to 40% alcohol, the amount of this protein precipitated is roughly proportional to the concentration of the alcohol. At the limits of 40 and 60%, distinct changes in the solubility are observed, and practically the same amount of nitrogen is precipitated. At the latter stage, both curves rise suddenly,

and run approximately parallel to the end. The latter part of the curve consists of the protein or proteins most difficult to precipitate by alcohol.

With a concentration of 55% and upwards, of alcohol, the gums present in the extract are precipitated with the protein, and their influence on the percentage of nitrogen in the latter part of the curve is noticeable. The two curves of precipitate and nitrogen, however, run approximately parallel throughout.

As will be seen from the foregoing Tables, in addition to the proteins existing in the saline extracts, there is a considerable amount of nitrogenous compounds in a form other than protein.

N in form of soluble proteins	40 %
N left in residue of seeds	15
N in non-protein form	45
% of the total N in the seeds	100

The following experimental studies are devoted to the elucidation of the nature of this 45% of the nitrogen of the same Acacia seeds. The solutions were prepared in different ways, and the results are all stated in per cent. of the non-protein nitrogen, taken as 100.

(1) *Preparation of the solution*—Method (*a*). The powdered seeds were extracted as completely as possible with cold water. After filtering, the liquid was concentrated on a water-bath to about a litre; the coagulated proteins were removed, and the remaining proteins precipitated by tannic acid. The latter reagent was removed from the solution by lead acetate, the lead by sulphuric acid and hydrogen sulphide, and the excess of the latter by a current of air.

Method (*b*). The seeds were extracted as completely as possible by hot water, and the solutions filtered. The combined fluids were concentrated, and poured into alcohol, making a solution of 80% in strength. After standing over night, the proteins were filtered off. The clear alcoholic fluid

was distilled, and the remaining aqueous solution diluted with water to a definite volume.

(2) *The solution tested by protein-reagents*:—

Millon's reagent,—very faint positive reaction after boiling.

Heller's nitric acid test,—nil.

Xanthoproteic, boiling nitric, then ammonia,—exceedingly faint yellow.

Ehrlich's diazo-reaction,—doubtful, perhaps slightly positive reaction.

Biuret test,—negative.

Salicyl sulphonic acid—negative.

Salicyl sulphonic acid—in filtrate after saturation with ammonium sulphate,—no ppt.

Lead acetate—no ppt.

Phosphotungstic acid,—large white ppt.

Mercuric nitrate—bulky white ppt.

The solution is, therefore, practically protein-free, and the reactions of Millon's and Ehrlich's solutions show that only a trace, if any, of tyrosin or histidin can be present. A large amount of basic substances is indicated by the precipitate with the alkaloidal reagent phosphotungstic acid; and this may, of course, include certain lesser polypeptides which do not contain a biuret reacting group.

(3) *The distribution of the nitrogen.*—A solution of the non-protein nitrogen compounds was prepared by method (a), and examined in the following manner:—

(i.) The total amount of nitrogen was ascertained.

(ii.) A portion was set aside under a bell-jar with milk of lime, for the Schlösing estimation of ammonia; while another portion was distilled with magnesia in a current of steam, and the ammonia collected in decinormal acid. The Schlösing method gave small and variable results, and was discarded. When the solution is boiled with magnesia, a very

slow evolution of ammonia begins; it continues slowly, hour after hour, and does not seem to come to a definite end in a reasonable time. After twenty minutes boiling, about 1 cc. of decinormal ammonia had collected; and, after four hours, 5 cc.; it was then distilling at the rate of 0·4 cc. per hour. (In a control, it was found that the whole of the ammonia from 0·6 gm. of ammonium chloride was distilled in less than twenty minutes, at the same rate of boiling; and required over 100 cc. decinormal acid.) One must conclude from this, that the ammonia does not all exist preformed in the solution, but is evolved from a substance which slowly decomposes by the action of magnesia, when boiled.

(iii.) The original solution was next treated with phosphotungstic acid, and the precipitate of basic compounds assayed for nitrogen. A phosphotungstic precipitation was also done on the solution, previously boiled with magnesia for four hours.

(iv.) The phosphotungstic filtrate from the former was distilled with magnesia, while a part was hydrolysed by boiling, for two hours, in 10% HCl, then neutralised, and distilled with magnesia.

(v.) The phosphotungstic filtrate from No. i., was examined for amino-groups by the sodium hypobromite method. The phosphotungstic acid was removed as barium salt, and excess of barium precipitated by carbon dioxide. The nitrogen evolved by the hypobromite was measured in a eudiometer.

The order of the experiments is indicated thus:—

Magnesia distillation (ii.) —> phosphotung. (iii.) —> sod. hypobromite (v.)

Phosphotungstic (iii.) —> { distillation (iv.) / hydrolysis and distillation.

The percentage of nitrogen obtained in each of the above determinations, is set out in the following table:—

Table vii.

	N.
(i.) Total nitrogen in the protein-free solution	100·0
(ii.) Ammonia evolved in the cold by lime water	2·8
,, distilled with magnesia	9·9
(iii.) Pptd. by phosphotungstic acid after distilln.	18·2
(v.) Sod. hypobrom. in phosphotung.-filtrate after distilln.	19·3
Undetermined ...	52·6
(iii) Phosphotungstic ppt. in orig. solution ...	20·2
(iv.) ,, filtrate distilled with magnesia	1·13
,, ,, hydrolysed and ,,	4·23

(4) The next series was carried out on a solution obtained by method (*b*), protein precipitated by alcohol.

The following diagram will serve to show the order of the experiments.

Hydrolysis and distn. —> —hydrolysis and distn. —> nitrous acid.
(with branches: nitrous acid. / phosphotung.)

(i.) The solution was hydrolysed by boiling in 8% hydrochloric acid, for two hours, neutralised, and distilled with magnesia, and titrated every hour.

Table viii.

After 1st hour	8·07 %
2nd ,,	0·66
3rd ,,	0·46
4th ,,	0·41
4 hours.	9·6 % of the N.

(ii.) The same solution was then hydrolysed a second time in 8% hydrochloric acid, for two hours, and the distillation with the magnesia repeated.

Table ix.

Distilled ½ hour	5·2 %
½ ,, longer	1·2
½ ,, ,,	0·8
½ ,, ,,	0·8
	8·0 % of the N.

(iii.) The above hydrolysis, with 8% HCl, was accompanied by a considerable blackening of the solution, due to the formation of melanoidin. After the first hydrolysis, the residue in the retort from the distillation was dissolved in sulphuric acid, and allowed to stand. The black precipitate was filtered off. It contained 4·8% of the nitrogen.

(iv.) The solution, after these two distillations, was now used for the estimation of amino-groups; and, first, the sodium hypobromite method was tried, with entirely negative results. That is, after acid hydrolysis and distillation of the ammonia formed, those substances are destroyed, which had previously liberated nitrogen gas with this reagent.

The nitrous acid method of Sachsse and Kormann was then applied.

The apparatus gave the theoretical yield of nitrogen from a sample of pure asparagin. Portion of the solution, after the first hydrolysis and distillation, was treated by the above nitrous acid process, and gave, after all the necessary corrections were made, 65·7% of the nitrogen. The solution, after the second hydrolysis and distillation, yielded, in the same way, 38·8% of the nitrogen.

(v.) After the first hydrolysis and distillation, the solution was treated with phosphotungstic acid for basic substances, and the precipitate contained 14·1% of the nitrogen.

The results obtained from this series of experiments may now be tabulated as under:—

TABLE X.

Total nitrogen in the protein-free solution	100
(i.) Hydrolysed and distilled with magnesia	9·6
(iii.) Melanoidin formed by above hydrolysis	4·8
(ii) Second hydrolysis, distilled with magnesia	8·0
(iv.) Nitrogen evolved by sodium hypobromite	nil
Half nitrogen evolved by nitrous acid	38·8
Undetermined N	38·8
(i.) First hydrolysis and distillation with magnesia	9·6
(v.) Precipitated by phosphotung. after hydrolysis and distilln.	14·1
(i.) First hydrolysis and distilln. with magnesia	9·6
(iv.) Half N evolved by nitrous acid after hydrol. and distilln.	65·7

(5) Seeing the effects of two successive hydrolyses, in decomposing certain of the constituents, to be so marked, the following series was designed to show the effect of varying the conditions of hydrolysis. For this purpose, a solution was obtained by the method described in paragraph 1 (b), and was further treated in the following manner. The liquid was made to contain 5% (by weight) of sulphuric acid, and a solution of phosphotungstic acid was added till no further precipitate formed on standing. The clear fluid was decanted, and the rest separated by the centrifuge, the precipitate being washed with the dilute reagents. From the fluid, the reagents were removed by baryta in slight excess, and the latter by carefully titrating with sulphuric acid, till the colour with phenolphthalein was just removed. After filtration, the clear pale yellow fluid was divided into five equal portions for hydrolysis, as follows:—

a. Boiled for 1 hour with 8% (by wt.) hydrochloric acid.
b. ,, 2 hours ,, 8 ,, ,,
c. ,, 4 ,, ,, 8 ,, ,,
d. ,, 8 ,, ,, 8 ,, ,,
e. ,, 15 ,, ,, 25 ,, sulphuric acid.

The hydrolysed fluids were then each neutralised, and distilled with magnesia by a current of steam into standard acid. The distillate was titrated after every hour, for four consecutive hours, and gave the following figures:—

TABLE XI.

	a.	*b.*	*c.*	*d.*	*e.*
After 1st hour ...	9·41%	10·38%	11·86%	13·73%	24·6%
,, 2nd ,,	1·00	0·86	0·72	1·29	3·2
,, 3rd ,,	0·72	0·43	1·00	1·00	2·3
,, 4th ,,	0·72	0·43	0·28	0·43	0·9
	11·85	12·10	13·86	16·45	31·0%

per cent. of the non-protein N in the extract.

Results.—In the above extract, after phosphotungstic acid precipitation, the process of hydrolysing decomposes some of the constituents, with the liberation of ammonia. The quantity of ammonia set free increases with the length of time the solution is boiled, and also with the strength of the

acid used. Secondly, the titrations, after distilling for four hours, are still yielding much ammonia, and, no doubt, would have continued for many hours longer, so that even the last result of 31% is not at all the maximum figure obtainable.

Thirdly, the ammonia which distils is not all preformed in the solution; after the first hour, all preformed ammonia must have been evolved, and the solution, by the continued boiling with magnesia, still liberates ammonia slowly, and in gradually lessening quantity. This is not characteristic either of amides or amino-acids.

However, the residual fluids in the distillation flasks were now examined for compounds possessing the amino-group. The apparatus for the nitrous acid method of Sachsse was used as in a former operation. In the majority of these determinations, no nitrogen was evolved. In a few, a small amount only was obtained, which would account for less than 0·5% of the non-protein nitrogen.

The same residual fluids, after distillation with magnesia, were next submitted to the Sörensen* titration.

In this, the solution is titrated with one-fifth normal sodium hydroxide, before and after treatment with neutral formaldehyde. In the first stage, free acid and also those carboxyl groups which are distant from an amino-group are neutralised; and, in the second stage, the formaldehyde removes the amino-group, and with it disappears the protective influence of the latter on the α carboxyl-group, thereby making the carboxyl-group available in the second titration.

Results.—Solutions *a*, *b*, *c*, *d*, *e*, when titrated, were found to be neutral. After addition of the formaldehyde solution, they still remained neutral. From these results we should conclude that compounds containing the amino-group were absent.

* Biochem. Zeitsch. 7, 1907, s.45.

The solid content of the protein-free extract.—A portion of No. 4 extract, after precipitation of the proteins with alcohol, was taken, to ascertain the amount of total solids present in it. Fifty cc. were evaporated *in vacuo* over sulphuric acid at ordinary temperature, and dried, over $CaCl_2$, in a vacuum desiccator, to constant weight. The residue was equivalent to 20%, by weight, of the seeds. A larger volume also was evaporated at a gentle heat to a syrup, then put aside for some time to crystallise. After the lapse of a few weeks, it was still a clear yellow syrup showing no signs of crystal-formation. However, on examining it some months later, with a lens, it was observed to contain numerous tyrosin-like clusters or groups of radiating needles. These minute crystals did not possess the opaque white appearance so characteristic of tyrosin, and gave a negative Millon reaction. The residue was then a tough and horny mass.

Another portion of the same solution, No. 4, was precipitated by mercuric nitrate. From the precipitate, the mercury was removed by hydrogen sulphide, and the liquid evaporated, at a very gentle heat, to a syrup, then set aside. After standing many weeks, no crystallisation had taken place, the substance remaining as a clear dark syrup.

A part of the same solution, No. 4, was then examined for lipoids, by shaking out with pure ether, a number of times. The ethereal liquid was dried with calcium chloride, then evaporated to dryness, and the residue weighed. It amounted to 0·546%, by weight, of the seeds. By assuming the whole of this ether extract to be lecithin (which contains 1·78% of N), the nitrogen required would be 0·0097, a quantity which amounts to just 0·48% of the non-protein nitrogen. It may, therefore, safely be concluded that the possible lecithin-nitrogen does not exceed 0·5% of the whole non-protein nitrogen present.

Discussion of Results.

i. The Proteins.

Solubility.—The seeds of *Acacia pycnantha* contain 4·5% of nitrogen, partly in the form of protein, and partly as other nitrogenous compounds. Table i. shows that, of the total nitrogen, over 70% can be extracted from the seeds by water, and 13% by sodium chloride. By extracting as completely as possible with 10% salt solution, and treating with a 5% tannic acid solution, the proteins precipitated correspond to 40% of the nitrogen, and the filtrate contains 45% nitrogen as non-protein compounds. The solubility of the proteins in salt-solution is greatly increased by nearly neutralising to phenolphthalein. In Table ii., are given the relative amounts of protein extracted by sodium chloride from 1 to 10%, alone, and with alkali added till nearly neutral.

	Alone.	With 0·1% alkali.
Sodium chloride 1%	5	8
10%	8	10
Sodium hydroxide 0·1%	8	

The same amount is extracted by 0·1% alkali as by 10% salt.

An extract of the seeds in 10% salt-solution filtered clear, slowly becomes acid to litmus, and deposits protein on standing. According to Osborne, an insoluble salt is formed of the basic proteins with the free acid of the extract.

Action of various precipitants.—From a 10% salt-extract, the following reagents precipitate the proteins in decreasing amounts, in the order given, and in the following relative proportions:—

Tannic acid	14· % of the total N
Trichloracetic acid	11·5
Heat coagulation	10·
Carbon dioxide	9·
Sodium chloride saturation	2·

A 5% tannic acid solution was added to the 10% salt-extract till no further precipitation took place, avoiding excess. The

solutions were kept cool by standing in water. This reagent precipitates the largest amount of protein, and the filtrates were biuret-free. With regard to the nature of this protein, the following authorities are quoted:—

Sebelien,* 1889, prepared proteins by salting out egg-albumin, casein, etc., and found that these were completely precipitated from solution by tannic acid, giving nitrogen-free filtrates. He used a solution of tannic-acetic acids and alcohol.

Effront,† 1899, showed that the end-products of peptic digestion of fibrin escape precipitation with tannic acid, and that, besides peptones, some albumoses remain in solution.

Neumeister‡ states that this reagent precipitates all proteins, including proteoses and peptones.

Simon,§ separated the total proteins of milk completely by a solution of tannic-acetic acids and alcohol, but found that, with tannic acid alone, quantitative results could not be obtained; also that good results ensued only when sufficient inorganic salts were present.

Hedin,‖ 1904, by using a tannin-salt-acetic acid solution, showed that the amount precipitated varied with the concentration of the protein-solution; and further, that the tannin-filtrate contained peptones and lower products of digestion.

Mack,¶ 1904, after preparing pure peptones by Siegfried's method, showed that they were precipitated from strong solutions by tannic acid, the precipitates being soluble in acetic acid.

Winterstein and Bissegger**, 1906, used the tannic-acetic acids alcohol-mixture to precipitate the total protein of cheese-extracts, and found that the results were influenced by the amount of

* Sebelien—Zeit. physiol. Chem. 13, 1889, 135.
† Effront—Chem. Ztg., 24, 1899, 770, 783.
‡ Neumeister—Lehrb. d. physiol. Chem. ii., 234.
§ Simon—Zeit. physiol. Chem. 33, 1901, 470.
‖ Hedin—Journ. Physiol. 30, 1904, 156, 195.
¶ Mack—Zeit. physiol. Chem. 42, 1904, 259.
** Winterstein u. Bissegger.—*ibid.* 47, 1906, 38.

tannic acid used, and that the presence of sodium chloride makes separation more complete.

Mey *, 1906, found, in the tannin filtrate from peptic digests, numerous peptone-like substances giving the biuret reaction.

Bigelow and Cook †, 1906, by numerous experiments, determined the conditions of maximum precipitation for Witte's peptone to be concentration of tannic acid 5%, sodium chloride 15%, in the final solution in which precipitation is made. This gave the maximum precipitation of proteins, and separated 94% of the nitrogen of Witte's peptone. This reagent precipitated proteoses and peptones at 12°C.

Suzuki ‡, 1907, employed a tannin-salt solution in his experiments on germinating seeds, to separate total proteins, including peptones.

Bialosuknia §, 1908, and numerous other workers in plant-proteins, measure the activity of proteolytic enzymes by the increase of nitrogen in the tannin filtrates.

These references suffice to show the uncertainty which existed with regard to the completeness of the precipitation by tannic acid. There is no doubt concerning the true proteins, as Sebelien showed, their precipitability being complete when the correct conditions, as to the amount of reagent and concentration of protein, are found for each case, conditions which vary with the nature of the protein. But, in dealing with protein-derivatives, there is now sufficient evidence to show, that many of the polypeptides are redissolved by excess of the reagent, so that the filtrates may give a positive biuret reaction. Since the polypeptides may exist in decreasing molecular magnitude, from the very complex to the simple dipeptides, it would appear useless to fix any limits as to which are, and which are not precipitated. But it is quite certain that the smaller members are soluble.

* Mey—*ibid.* 48, 1906, 81.

† Bigelow & Cook—Journ. Amer. Chem. Soc. 28, 1906, 1485.

‡ Suzuki—Journ. Biol. Chem. iii., 1907, 268.

§ Bialosuknia—Zeit. physiol. Chem. 58, 1908, 487.

The trichloracetic acid figure is less than that for tannic acid. Most proteoses and peptones are soluble in this reagent. Distinct evidence of proteoses is given in Table iv., by the deposit formed on cooling the hot filtrate; this deposit redissolves on heating. Proteoses are only partially precipitated by excess, dissolve on boiling, and re-appear on cooling, while peptones are not precipitated.*

The protein coagulated by boiling the salt extract, slightly acidulated with acetic acid, is considerably less than the tannin-precipitate. The results of Osborne, Chittenden and Mendel†, show that coagulation of reserve-proteins of seeds is always incomplete, and that their behaviour is wholly different from that of animal-proteins. On this account we cannot designate the uncoagulable protein, which is precipitated by tannic acid, as proteose and peptones, which is so often done in the separation of animal-proteins.

Carbon dioxide precipitates a little over one-half (63%) the amount obtained by tannic acid. This probably represents the actual globulins present.

A very small quantity only is obtained by complete saturation with sodium chloride.

By fractional salting out with ammonium sulphate, precipitation begins with six-tenth's saturation. The globulins, which are most readily salted out, appear first; and since we have seen that carbon dioxide shows the presence of at least 63% of globulins, they must constitute the whole of the seven-tenth's fraction, and part of the eight-tenth's. All the work done on the globulins, up to within a few years ago, was based on the fact that globulins were defined as those proteins which could be salted out by half-saturation with ammonium sulphate—a definition very far removed from the truth, and according to which the extract would contain no globulins at all.

* Martin, C. J., Journ. Physiol. 15, 1894, 375.
† Journ. Physiol. 17, 1894, 48.

By quantitative precipitation with alcohol, in increasing successive concentrations, a differentiation occurs into at least two distinct proteins. The precipitation of the first runs approximately proportional to the concentration of alcohol, from the commencement to 40%. From 40 to 60% the nitrogen is nearly constant, and a second protein is indicated at 60% concentration, by the sudden change in the solubility of the precipitate, and the increase in amount of nitrogen.

ii. The non-protein Nitrogen Compounds

The experimental work may be grouped under the following headings:—

A.—Preparation of a protein-free solution containing other nitrogen compounds, and proof of absence of protein.

B.—Distillation of free ammonia in the solution.

C.—Hydrolysis by dilute acids under a reflux condenser, and subsequent distillation with magnesia, by Sachsse's method for amides.

D.—Continued and drastic hydrolysis, involving decomposition of substances which yield more ammonia than C.

E.—Precipitation of basic constituents with phosphotungstic acid.

F.—Examination for compounds containing the amino-group.

A.—The non-protein nitrogen solutions are obtained by two methods. (a) Cold saline extracts are precipitated by tannic acid, lead acetate, and hydrogen sulphide. (b) Hot distilled water extracts are precipitated in 80% alcohol. The alcohol containing the non-protein constituents is distilled under reduced pressure, the aqueous residue diluted with water, and filtered. Evidence is shown that these solutions are practically protein-free. The solutions contain no nitrates and no alkaloids, and when distilled no nitrogen is found in the distillate.

B.—Ammonia is obtained from all the extracts by distilling with magnesia in a current of steam. Zymolysis during the extraction of the seeds, with its consequent liberation of ammonia, is entirely excluded in method (b) by boiling. That this ammonia is actually free in the extracts, is doubtful, since the desamidising enzymes of seeds do not become active till germination begins. There remains then the probability that compounds are present, which decompose with great ease by distilling with magnesia. In support of this, we have (in Table vii., iv.) the result of a distillation, following the removal of all pre-existing ammonia, and other basic compounds, with phosphotungstic acid: ammonia is formed as before. Again, when distilled directly (Table vii., ii.), and after hydrolysis with dilute acid (Table x., i.) practically equal quantities are obtained in the same time.

C.—By hydrolysing with dilute acids, amides split off ammonia with great readiness, which distils off rapidly with magnesia. In Tables viii. and xi.*a*, the minimum figures are obtained under conditions well known to yield the whole of amide-ammonia. These are about 8-10% of the non-protein nitrogen, and would represent about 2% of asparagin in the seeds. On the other hand, when the attempt is made to isolate amides by Schulze's method with mercuric nitrate, only a syrupy residue is left, which shows no crystallisation on long standing. Again, the magnesia distillations, instead of coming to a sharp finish, apparently go on for some considerable time, evolving ammonia (Table xi.), as if it were gradually formed by the slow decomposition of substances other than amides. In consequence, the invariable procedure of ascribing to amides, this ammonia obtained by Sachsse's process, can certainly not be applied here.

D.—By increasing the duration of hydrolysis, and strength of acid, the decomposition is accelerated, with an increased liberation of ammonia. Boiling with 25% sulphuric acid, for 15 hours, results in the formation of 31% of ammonia-

nitrogen. Even after this powerful treatment, the ammonia distillation is drawn out for some hours, as is seen in Table xi., *e*.

E.—The phosphotungstic precipitates, when dissolved, and the reagent removed with barium hydroxide, begin rapidly to decompose; and, within a few days, considerable volumes of trimethylamine are liberated. This strong evidence of the presence of cholin is partly confirmed by subsequently obtaining the characteristic haemin-like crystals of cholin per-iodide, which are recognised under the microscope in abundance. That this free cholin has not its origin in lecithin or other lipoids occurring in the extract, is seen from the small amount of lecithin, 0·5%, obtained by ether-extraction. Both cholin and betain have been identified by Schulze in leguminous seeds.

The same solution gives, with silver sulphate, a considerable precipitate containing xanthin-bases, and, after saturation with baryta, a precipitate which probably contains arginin. The total nitrogen-value of these basic compounds is 20% of the non-protein nitrogen, but when the phosphotungstic precipitation follows hydrolysis, only 14% is obtained. The difference is mostly accounted for by the formation of a large amount of melanoidin, which is explained by Samuely* as probably due to the association of the nitrogenous compounds with carbohydrates present in the solution, and their oxidation during the hydrolysis with acid. Schmiedeberg† noticed also that xanthin bases and carbohydrates gave rise to melanoidin, when boiled with acids.

F.—In the examination of the solution for compounds containing the amino-group, the following results were obtained:—

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* Hofmeister's Beiträge, 1902, s.355.

† Arch. f. exp. Path. u. Pharmak., 43, 1899, s.57.

	Sodium hypobrom. N.	Nitrous Acid N.
After :—		
1. Magnesia dist. + phosphotung. acid.....	19·3 %	
2. Hydrolysis and distillation.....	nil	65·7
3. Hydrolysis + hydrolysis and distilln...		38·8
4. Phosphotung. + hydrolysis and distilln.		0--0·5
	% of the	non-prot. N.

Of the compounds known to evolve nitrogen gas with sodium hypobromite, ammonia and basic compounds are excluded in 1, and only certain amides are left to represent the 19·3% of nitrogen. In 2, amides also are excluded and no nitrogen was obtained. Although the two plant-amides, asparagin and glutamin, do not evolve ammonia with hypobromite till hydrolysed with dilute acids, yet this does not exclude the existence of other compounds in which the amino-group is in a less stable position, and which would evolve ammonia, like urea, allantoin, etc., with this reagent. It is significant that the nitrogen in 1, and that obtained by Sachsse's method after prolonged hydrolysis (Table xi. *d*), and which has been already discussed as a possible amide-figure, are approximately the same. This reagent does not liberate nitrogen from amino-acids.

Nitrous acid, on the other hand, decomposes almost all amino-groups with evolution of nitrogen. After hydrolysis, such compounds must be present, representing the high figure in 2. After a double hydrolysis, the nitrogen evolved by nitrous acid is reduced to about one-half, and, following phosphotungstic acid and hydrolysis, no nitrogen is obtained.

Amino-acids.—Van Slyke* found that no nitrogen was evolved from prolin or glycin anhydride which contain the imino-group; also that guanidin, creatin, and the amide-group of asparagin, do not react. Slyke and Hart† have shown that amino-acids, boiled with magnesia, do not evolve

* Journ. Biol. Chem. 7, 1910, p. xxxiv.

† Amer. chem. Journ. 29, 1903, 168.

ammonia. Kruger and Schmid* heated amino-acids with concentrated sulphuric acid at 160° C., and showed that no ammonia was given off on distillation with alkali. Now 2 and 3 show that the nitrogen is obtained from compounds which are rapidly decomposed by hydrolysis, and 4 that, after removal of basic compounds, the hydrolysis and distillation remove these readily decomposable compounds almost entirely. We can, then, only conclude from the above that amino-acids are not present, or exist in very small amounts (See 4) in the non-protein nitrogen solution. This conclusion is confirmed also by the formaldehyde titration.

This slow decomposition, with formation of ammonia, is characteristic of certain groups of organic compounds. Erdmann † has shown that compounds containing the nitril grouping, when heated with sulphuric acid, form amines, and finally ammonia.

Embden‡ found that cystin gives off ammonia, when boiled for a long time with magnesia; and Mathews and Walker§ that it oxidised spontaneously in alkaline solutions, setting free ammonia. Neuberg and Mayer‖ crystallised cystin in radiating bunches of needles like tyrosin.

Jolles¶ by slow oxidation of plant-protein, at ordinary temperature, obtained urea 50%, nitrogen in phosphotungstic acid precipitate 20%, and in filtrate 30% of the total nitrogen. The urea originates in the—CO·NH—and—$CO{\cdot}NH_2$ groups of the protein molecule, and is analogous with the breaking down and oxidation of proteins in the organism.

Plimmer**, by oxidising albumins, obtained hydrocyanic acid, and he states that it arises from the glycin and aspartic

* Zeit. physiol. Chem 30, 1900, 556.

† Erdmann—Journ. Biol. Chem. 8, 1910, 41.

‡ Embden—Zeit. physiol. Chem. 32, 1900, 95.

§ Mathews & Walker—Journ. Biol. Chem. 6, 1909, 289

‖ Neuberg & Mayer—Zeit. physiol. Chem. 44, 1905, 472.

¶ Jolles—*ibid.* 32, 1900, 361.

** Plimmer—Journ. Physiol. 32, 1904, 51.

acid. Maly* and others also obtained oxidation-products of proteins, which were not precipitated by tannic or phosphotungstic acids, gave no Millon or xanthoproteic test, but a positive biuret. These products, heated with alkali, evolved large amounts of ammonia; after hydrolyses, they yielded amino-acids and ammonia, and gave off nitrogen when acted on by nitrous acid.

Plants and seeds contain protease and oxidase ferments, and, therefore, it is not improbable that the above oxidation-products are present in the non-protein solution.

Polypeptides.—Swirlowski† submitted protein to hydrolysis with 0·5% hydrochloric acid, at 37° C for six months. The phosphotungstic filtrate then contained 27% of the nitrogen, and no amino-acids could be obtained, until hydrolysed by strong acids. These polypeptides gave only the biuret reaction.

We have now seen that both tannic and phosphotungstic acids may not precipitate the smaller polypeptides. If we assume the presence of these in the Acacia solutions, then (1) we know, from the negative Millon test, that the tyrosin nucleus is not a constituent. (2) It is more difficult to explain the absence of the biuret reaction; though it is just possible that the biuret-yielding group is absent, it is more likely that, with these particular polypeptides, the reaction is not reliable. (3) By ordinary hydrolysis, only small amounts of ammonia would be set free, certainly not sufficient to account for the large amount obtained. On the other hand, if oxidation has also taken place, then, as has already been shown, oxidation-products could be slowly formed, which would provide large amounts of ammonia on distillation. (4) By the severing of imino-linkings in the polypeptide hydrolysis, amino-groups would certainly appear,

* Maly—Sitzber. Wien. Akad. 1889; Monatshr. 1889.
† Zeit. physiol. Chem. 48, 1906, 252.

which ought to be detected by the nitrous acid method. The latter, however, gives no nitrogen after the phosphotungstic precipitation and hydrolysis, and this result may be interpreted, either as proof of the absence of polypeptides, or that, by steric hindrance, the reaction is made exceedingly slow.